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Antibacterial activity of *Piper betle* ethanolic extract against ESBL producing *Escherichia coli* in sepsis induced BALB/c mice

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Abstract

Sepsis is an organ dysfunction caused by dysregulation of immune response towards systemic infection with high incidence and mortality rate. Innovative breakthrough is required to manage sepsis especially extended spectrum beta lactamase (ESBL) producing *Escherichia coli* (*E. coli*) induced sepsis. Utilization of *Piper betle* extract to manage sepsis could be considered as an alternative therapy and should be proven scientifically so meropenem utilization could be reduced. This study aimed to evaluate the antimicrobial effect of *Piper betle* ethanolic extract in BALB/c mice infected with ESBL producing *E. coli*. We identified terpene, apiol, polyphenol, eugenol, myristicin, flavonoid, safrole, lynalil acetate, and allyl tetramethoxy benzene in the *Piper betle* ethanolic extract using thin layer chromatography. The minimum inhibitory concentration of the *Piper betle* ethanolic extract against ESBL producing *E. coli* was 3 mg/mL assessed using broth dilution method. We evaluated the effect of *Piper betle* ethanolic extract to the survival rate of BALB/c mice infected with ESBL producing *E. coli*. The single dose administration of *Piper betle* ethanolic extract prolonged the survival rate of the infected BALB/c mice for 6 hours.

Keywords: *Piper betle* ethanolic extract; ESBL producing *Escherichia coli*; Sepsis induced mice; Survival rate.

1. Introduction

Sepsis was defined as life threatening organ dysfunction caused by dysregulation of immune response towards systemic infection [1]. Sepsis incidence is about 66-300/100000 population number with mortality rate 27-36% in developed countries while in developing countries, the mortality rate of multiple organ dysfunction due to sepsis was 70% [2, 3].

The most common bacteria causing sepsis is *Escherichia coli* (*E. coli*). The emergence of extended spectrum beta lactamase (ESBL) producing *E. coli* leads to the therapeutic failure and excess mortality [4, 5]. The previous studies reported that the incidence of ESBL producing *E. coli* infection in England was 10%, there were 925 cases of ESBL producing *E. coli* infection in USA noted by The Duke Infection Control Outreach Network and there were 883 cases of ESBL producing *E. coli* infection in Thailand and the incidence tends to increase every year. The association between high incidence of ESBL producing *E. coli* infection with misused of antimicrobial therapy was described in the multiple studies [6, 7, 8, 9].

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Meropenem is widely used as the antibiotic therapy for sepsis due to ESBL producing *E. coli* and then the resistance to meropenem become the problem globally. The active compounds of *Piper betle* leaves extract such as flavonoid and eugenol, have potential effect to disturb the growth of Gram negative bacteria including *E. coli* [10, 11]. Therefore, the use of *Piper betle* leaves extract as antimicrobial therapy for ESBL producing *E. coli* infections was considered as an innovative breakthrough [12]. Little is known regarding the antimicrobial effect of *Piper betle* extract towards ESBL producing *E. coli.* This study aimed to evaluate the antimicrobial activity of *Piper betle* extract towards ESBL producing *E. coli* in sepsis induced BALB/c mice.

2. Material and methods

The ESBL producing *E. coli* isolate provided by the Department of Clinical Microbiology, Faculty of Medicine, Brawijaya University was identified and confirmed by Vitek-2 system and double disk synergy test, respectively [13].

2.1. Plant material and extract preparation

The *Piper betle* plant was obtained and confirmed by a plant taxonomist in UPTD Materia Medica, a medicinal plants cultivation center, in Batu City, Indonesia. We selected five of *Piper betle* leaves located on the tip of the plant shoot system for preparing the extract. The leaves were dried under shade for 5 days and ground using a blender into a coarsely dry powder followed by ethanolic maceration as previously described [14]. Briefly, one gram of the *Piper betle* leaves powder were macerated in 20 ml 96% ethanol for 6 hours at room temperature. Then, the filtrate was collected using filter paper Whatman 40 and washed for three times using sterile aquadest. The solvent was evaporated in the oven at 100 ℃. The extract was stored in 4℃refrigerator until use.

2.2. Active compound identification

The active compounds of *Piper betle* leaves extract were determined using thin layer chromatography as described by Valle Jr et al, 2016 [15]. The Rf values of chromatograms were used to determine the active compounds based on Rf values in the references [16, 17].

2.3. Antimicrobial sensitivity test of *Piper betle* ethanolic extract

Broth dilution method was conducted to identify the Minimal Inhibition Concentration (MIC) of *Piper betle* leaves extract against ESBL producing *E. coli* [18]. Six concentrations of *Piper betle* leaves extract including 0 mg/mL; 1 mg/mL; 2 mg/mL; 3 mg/mL; 4 mg/mL; and 5 mg/mL were prepared in Mueller Hinton broth medium. After overnight incubation at 37℃, the MIC was observed and then ten micro liters of bacterial inoculum of each concentration were inoculated on to the Mueller Hinton agar medium and incubated at 37℃. The number of bacterial colonies growing on the Mueller Hinton agar medium was counted after 24 hours incubation.

2.4. Animal infection and survival rate evaluation

Female BALB/c mice between 6-8 weeks of age were obtained from Bioscience Laboratory, Universitas Brawijaya, Malang, Indonesia and used in this study. The mice were divided into three groups (9 mice per group): (i) negative control group including mice that received *Piper betle* leaves extract orally with the dose corresponding to the MIC; (ii) positive control group including mice infected with 250 µL of 10^9 CFU/mL ESBL producing *E. coli* suspension through intraperitoneal injection to induce sepsis; (iii) treatment group including mice infected with 250 µL of 10^9 CFU/mL ESBL producing *E. coli* suspension through intraperitoneal injection treated by *Piper betle* leaves extract orally with the dose corresponding to the MIC 30 minutes after bacterial injection. The animals were monitored every 6 hours daily up to 96 hours. The *in vivo* dose of the extract was converted from the MIC value multiplied by a total blood volume of mice. This study was approved by Health Research Ethics Committee Faculty of Medicine, Brawijaya University (172/EC/KEPK/10/2020).

2.5. Statistical analysis

The number of bacterial colonies obtained from antimicrobial susceptibility test of *Piper betle* leaves extract against ESBL producing *E. coli* was analyzed statistically using One Way Anova followed by simple linear regression analysis. The survival rate data was analyzed using Kaplan Meier analysis. The value less than 0.05 is considered significant.

3. Results and discussion

3.1. The active compounds of *Piper betle* leaves extract

There are nine active compounds identified in *Piper betle* leaves extract by thin layer chromatography, however only four active compounds are known to have antimicrobial effects including terpene, eugenol, polyphenol and flavonoids (Table 1).

Table 1 The active compounds of *Piper betle* leaves ethanolic extract

Rf value	Active Compound	Roles or Effects
0.1348	Lynalil Acetate[16]	Decongestant [16].
0.2586	Terpene [16]	Aromatic compound, antimicrobial [12, 19]
0.4323	Allyl tetramethoxy benzene[16]	Major component of essential oil [16].
0.5007	Eugenol [16]	Antiseptic and anti-inflammatory [12].
0.7271	Apiol [16]	Food additive [16]
0.8309	Myristicin [16]	Anti-inflammatory, cytochrome P450 enhancer [20]
0.8790	Polyphenol [17]	Antioxidant, antimicrobial [16, 21]
0.9280	Flavonoids [17]	Heart stimulant, diuretic, antimicrobial [12, 21].
0.9554	Safrole [16]	Component of candy and beverage [12].

3.2. The antimicrobial effect of *Piper betle* ethanolic extract towards ESBL producing *E. coli*

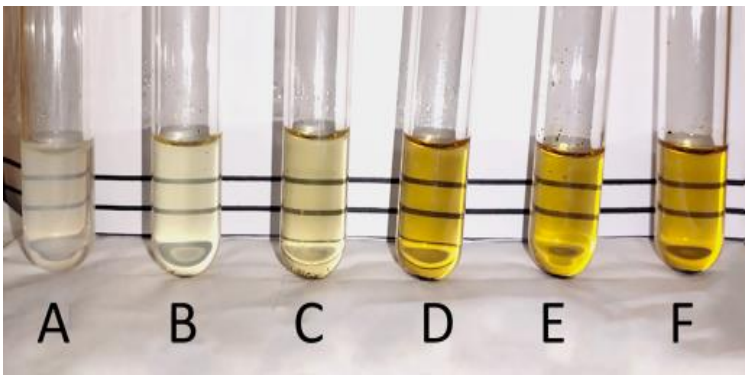

Figure 1 with the broth dilution method of *Piper betle* leaves extract against ESBL producing *E. coli*
(A: 0 mg/mL; B: 1 mg/mL; C: 2 mg/mL; D: 3 mg/mL: E: 4 mg/mL; F: 5 mg/mL)

We identified the MIC value of *Piper betle* leaves ethanolic extract against ESBL producing *E. coli* was 3 mg/mL,. The increasing of the concentration of *Piper betle* leaves ethanolic extract was followed by a decrease of number of colonies growing on the Mueller Hinton agar ($p<0.001$). However, the linear regression analysis showed weak association between *Piper betle* leaves ethanolic extract in inhibiting the growth of ESBL producing *E. coli* in vitro indicated by the value beta-coefficient of -0.666 (Table 2).

Table 2 The number of colonies of ESBL producing *E. coli* after being exposed by *Piper betle* leaves ethanolic extract

Concentration (mg/mL)	Plate A (10^7 CFU/mL)	Plate B (10^7 CFU/mL)	Plate C (10^7 CFU/mL)	Mean± SD (10^7 CFU/mL)
0	26,508	29,507	36,316	30,777± 5,026
1	20,914	20,590	23,184	21,563± 1,413
2	8.6	7.4	10.3	8.8± 1.5
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0

The *in vivo* study showed that the survival rate of the treatment group was 6 hours longer than the positive control group (Figure 2).

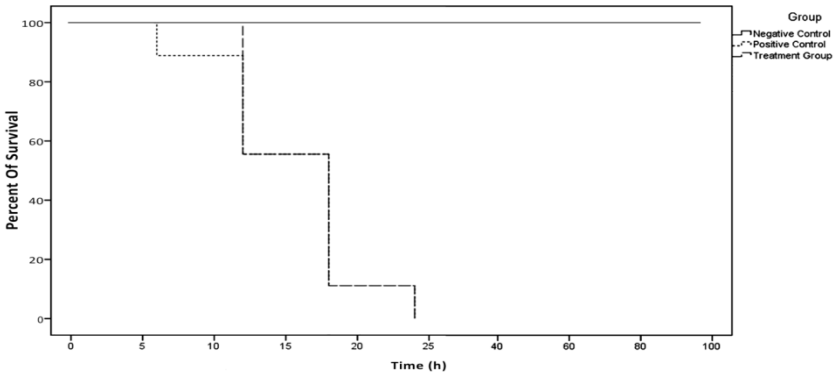

Figure 2 The survival rate of sepsis induced BALB/c mice by ESBL producing *E. coli*

Kaplan Meier analysis revealed single administration of 3 mg/mL *Piper betle* ethanolic extract 30 minutes after the mice were infected did not affect the survival rate of animal model significantly with p-value 0.960.

Several studies regarding *Piper betle* extract have proven its anti-cancer, antimicrobial, and antioxidant properties [22]. It is also known for its anti-inflammatory properties proven in animal models [23]. Das *et al*, in 2016, mentioned 35 active compounds found in *Piper betle* extract [12], with α-pinene, sabinene, eugenol, B-selinene, germacerene-B, spathuleneol, globulol, and allyl pyrocatechol diacetate having antimicrobial activities. Others active compounds like caryophyllene, A-humulene, and 1, 8-cineol have anti-inflammatory properties [12]. In this study, 9 active compounds were found. Terpene, eugenol, polyphenol, and flavonoids are known for their antimicrobial activities and have role in disrupting the growth of ESBL producing *E. coli*. Pungent scent of *Piper betle* ethanolic extract is produced by terpene as major component of aromatic compound. Oily texture of the extract is produced by allyl tetramethoxy benzene which is a major constituent of essential oil.

Antimicrobial effect of *Piper betle* extract has been evaluated in several bacteria such as *Streptococcus pyrogenes*, *Staphylococcus aureus*, *Proteus vulgaris*, *E. coli*, *Pseudomonas aeruginosa, Enterococococcus faecalis*, *Citrobacter koseri*, *Citrobacter fruendi*, and *Klebsiella pneumoniae* [24]. Phumat *et al*, 2017, determined 1 mg/mL as the MIC of *Piper betle* ethanolic extract towards *Streptococcus gordoni* and 2 mg/mL as the MIC of *Piper betle* ethanolic extract towards *Streptococcus mutans* [25]. In this study, the MIC was 3 mg/mL, higher than aforementioned study however the linear regression analysis showed weak antimicrobial effect of *Piper betle* ethanolic extract against ESBL-producing *E. coli*. We suggested that higher concentration of *Piper betle* ethanolic extract was required to disrupt the growth of highly resistant bacteria. It is also worthy to notice that Streptococcal bacteria is Gram positive bacteria while *E. coli* is Gram negative bacteria. Perhaps, *Piper betle* ethanolic extract is more potent disrupting the growth of Gram positive bacteria compared to Gram negative bacteria.

Survival of sepsis induced mice varies in many studies. A study in Southern Medical University, Guangzhou China, showed decreased survival rate of infected mice up to 34%, 60 hours after intraperitoneal injection of *E. coli* [26]. Another study from Universidade Ceuma in Brazil showed decreased survival rate of infected mice up to 34% during 12 hours period after intraperitoneal injection of *E. coli* and 100% mortality after 24 hours [27]. In this study, the survival rate of mice in positive control group decreased into 55.6% 12 hours after bacteria injection and after 18 hours post injection, the survival rate was 11.1%. Similar to the previous study, all mice in positive group control were death 24 hours after bacterial injection. This result is not significantly different compared to treatment group. As our knowledge, there was no other study describing the effect of *Piper betle* extract towards sepsis induced mice, however Chauhan *et al*, 2019, describe significant improvement of sepsis induced mice in isorhamnetin treated group [28]. Another study revealed that all treated group mice survived during observation period [28], however the survival rate of treated group mice was longer only 6 hours compared to positive control group. Several factors such as bioavailability, metabolism, and excretion may affect the effect of the extract administered orally. Hence, the *Piper betle* ethanolic extract levels in the mice body will not be sufficient to overcome the sepsis.

There are some limitations of this study. First, the number of colonies of ESBL producing *E. coli* decreased after being exposed by 3 mg/mL of *Piper betle* ethanolic extract. Narrowing the dose interval of the extract might generate more exact MIC and MBC value and better statistical analysis. Second, single dose administration of the *Piper betle* ethanolic extract may affect short survival rate of the mice. Multiple doses administration of the extract may prolong the survival rate.

4. Conclusion

This study proved the antimicrobial effect of *Piper betle* ethanolic extract was capable to disrupt ESBL producing *E.coli* in vitro model with MIC 3 mg/mL, but single administration of *Piper betle* ethanolic extract was not sufficient to increase the survival rate of sepsis induced mice. Further investigation is needed to evaluate the effectiveness of multiple administration of *Piper betle* ethanolic extract at certain intervals.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All authors declare no conflict of interest.

Statement of ethical approval

The study was approved by the Health Research Ethics Committee Faculty of Medicine, Brawijaya University (172/EC/KEPK/10/2020), and the methods were carried out in accordance with the approved guidelines.

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